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INTRODUCTION

Much of what we know about the details of neoplastic transformation comes from studies in cell culture. With the original demonstration by Weinberg, Wigler, and Barbacid that rodent cells could be transformed *in vitro* by DNA sequences from cancer cells, laboratory mouse strains and resultant cell lines have become premier models for the study of oncogene and tumor suppressor function. This has evolved in recent years to the study of genetically defined mouse cancer models, both transgenics and knock-outs. These have provided a great deal of key information regarding the transformation process and have served as model systems in which to test new anti-cancer therapies. Despite the utility of the aforementioned approach, there is a fundamental problem with absolute reliance on this paradigm. Genetic alterations that easily transform rodent cells in culture do not have a similar effect on normal human cells. This implies an inherent difference between the processes of neoplastic transformation in man and mice. In the face of intense scrutiny, the nature of this difference has remained a mystery for more than 30 years.

One distinguishing feature of primary rodent cell cultures is that they can be easily immortalized (Blasco et al., 1997a). Human cells, in contrast, rarely undergo spontaneous immortalization (Imam et al., 1997). This almost certainly reflects a difference in the mechanisms by which replicative lifespan is extended. In mouse cells, inactivation of the p53 pathway is sufficient for immortal growth, perhaps indicating that a damage response limits proliferation, whereas the process in human cells is much more complex (Chin et al., 1999). This most likely reflects a physical limitation on the proliferation of primary human cells that is imposed by their shorter telomeres and a profound lack of telomerase. This observation is further bolstered by the fact that the activation of telomerase is sufficient to immortalize primary human fibroblasts in culture (Counter et al., 1998).

Recent reports from the Weinberg lab (Hahn et al., 1999; Elenbaas et al., 2001; Lundberg et al., 2002) indicated that telomerase expression may serve as the critical difference between transformation of mouse and human cells. In these studies, it was shown that the telomerase catalytic subunit, hTERT, could cooperate with combined expression of the SV40 early region and activated Harvey Ras (V12) to transform primary human cells. Our data, however, indicate that this is not keystone to transformation of all cell types. Instead, we have shown that primary human fibroblasts can initially undergo transformation in the absence of telomerase and that telomerase activation typically occurs in the latter stages of tumorigenesis (Seger et al., 2002).

This work has provided important information regarding our understanding of the initiation and development of human cancers. Chiefly, this work has resulted in a minimal formula for transforming primary human diploid fibroblasts and human mesodermal cells. These experiments have also provided an opportunity to examine the precise combination of cellular pathways that must be altered in order to result in transformation. An additional benefit is that these cells engineered with precise genetic alterations serve as powerful tools for testing drug therapies, both *in vitro* and in xenograft models.

Ultimately, our understanding of a cancer cell serves two purposes. First, the study of transformation pathways has and will continue to yield important information

regarding how normal cells regulate growth and proliferation. Second, only through understanding the precise molecular events that are required to generate a human tumor may we hope to design rational and specific therapies to battle human cancers.

BODY

The field of cancer biology continues to grow and evolve through the development of new molecular tools and mouse model systems that are used to enhance our understanding of how oncogenes and tumor suppressors work within biological systems to promote tumorigenesis. Cell culture experiments utilizing both human and rodent cells have served as one of the central approaches to determining how these molecular networks interact during tumorigenesis, however, one must also note that these systems are not always directly comparable or fully representative of physiological conditions. One key example of such a mouse versus human difference is the number of genetic alterations required to convert a normal cell into a cancer cell. While rodent cells can be transformed by two genetic "hits," such as SV40 Large T-antigen or adenoviral E1A in combination with constitutively activated ras (Land et al., 1983; Ruley, 1983), human cells cannot be transformed by these combinations, indicating that the pathways involved in transformation may differ between mouse and human.

A significant and notable difference between rodent and human cells is the requirement for telomerase, the enzyme responsible for maintaining the ends of chromosomes, known as telomeres, during DNA replication. While rodent cells have constitutive activation of the telomerase enzyme in both somatic and germ cell lineages, human cells only have expression in germ cells (reviewed in McEachern et al., 1994). Interestingly, the majority of human tumors are telomerase positive (Kim et al., 1994), indicating a potential role for telomerase activation in tumorigenesis. In a series of publications, Weinberg and colleagues have shown that primary human fibroblasts, mammary epithelial, and small airway epithelial cells can be transformed by coexpression of the SV40 early region (large T-antigen and small t-antigen), the catalytic subunit of telomerase, hTERT, and a constitutively activated form of Harvey Ras (V12) (Hahn et al., 1999; Elenbaas et al., 2001; Hahn et al., 2002; Lundberg et al., 2002).

While the majority of human cancers indicate increased ras expression (typically through an activating mutation), recapitulation of this event has proven difficult in both rodent and human primary cell culture systems, as ras overexpression initiates a severe and irreversible growth arrest program called senescence (Serrano et al., 1997). While several proteins have been shown to bypass ras-induced senescence in primary mouse embryonic fibroblasts (E1A, Bmi-1, Tbx-2, and DRIL1) (Serrano et al., 1997; Jacobs et al., 1999; Jacobs et al., 2001; Peeper et al., 2002), only the adenoviral oncoprotein E1A has been shown to be effective of bypassing this effect in primary human fibroblasts (Serrano et al., 1997). It is also interesting to note that primary human skin fibroblasts (BJ, WI-38, DET551, and HS68) co-expressing E1A and Ha-RasV12 are capable of *in vitro* transformation in an assay for anchorage-independent growth (Seeger et al., 2002). These E1A + Ras expressing cells, however, do not result in tumorigenesis in immunocompromised mice, and require a third genetic alteration, overexpression of MDM2, to result in *in vivo* transformation (Seeger et al., 2002).

In the first year report for this project, results pertaining to the characterization of the E1A + MDM2 + Ha-RasV12 model of human cell transformation (Task #1) were outlined. These results culminated in a peer-reviewed publication in November 2002. In the second year report, we began to focus on a series of experiments that examined the functional interactions required for E1A-mediated transformation processes. These

experiments were conducted in a two-pronged manner. The first series of experimentation focused upon testing the ability of a small subset of genes to complement and rescue transformation defects of specific E1A functional mutants. The second approach was to screen a library of genes represented by short-hairpin RNA constructs (shRNAs) for gene silencing capable of complementing this same battery of E1A functional mutants. In this final report, I will present some of the preliminary data in these shRNA complementation experiments that will serve as the basis of continued work on human cell transformation models.

TASK #1: MODELS FOR HUMAN CELL TRANSFORMATION

As has been indicated in previous reports for this award (2002 and 2003), the goals of this task are now complete and resulted in a publication, "Transformation of normal human cells in the absence of telomerase activation" (*Cancer Cell* 2: 401-413 [2002]).

TASK #2: GENETIC REQUIREMENTS FOR HUMAN CELL TRANSFORMATION MEDIATED BY ADENOVIRUS E1A

The work contained within this task had been the focus of the experiments reported within the 2003 report, and continued to be a focus of experimentation conducted within the final year of this award. The bulk of the data reported in the 2003 report centered upon classical oncogene complementation experiments of two well-characterized regions of E1A: the 26-35 region of the N-terminus responsible for binding the transcriptional coactivator family, p400/TRRAP, and the highly conserved Rb binding motif, CR2.

The 26-35 region has been shown to be critical for E1A to interact with the p400 and TRRAP transcriptional coactivators. Interestingly, these coactivators were originally discovered through their interactions with the c-Myc oncogene. Therefore, I tested the ability of c-Myc to rescue the transformation defect of fibroblasts coexpressing E1A Δ 26-35 + Ha-RasV12. As was reported last year, fibroblasts coexpressing E1A Δ 26-35 + c-Myc + Ha-RasV12 are capable of robust anchorage-independent growth in soft agar. The ability of these cells to promote tumorigenic growth *in vivo* in immunocompromised mice has been tested repeatedly, yielding rather inconsistent results. In some mice, these cells were capable of promoting tumor formation, yet in some rounds of injections, these cells were incapable of promoting tumorigenesis, even after an extended latency. One potential explanation for these mixed results is that perhaps some E1A Δ 26-35 + c-Myc + Ha-RasV12 expressing cells were sensitized to apoptotic stimuli due to differences in c-Myc expression (a constant caveat in utilizing retroviral gene transduction for overexpression experiments), and this hypothesis is currently being tested.

The CR2 region is responsible for binding the RB tumor suppressor, the phosphorylation status of which regulates E2F-mediated transcription, and thus progression of the cell cycle to S phase. The complex of cyclin E and CDK2 is responsible for this hyperphosphorylation status of Rb. Studies of the pathways involved in E1A-mediated apoptosis indicate that E1A Δ CR2 can be functionally complemented cyclin E expression (A. Samuelson and S. Lowe, personal communication.) We therefore

tested the ability of cyclin E overexpression to rescue the transformation defect of E1A Δ CR2. Surprisingly, human fibroblasts coexpressing E1A Δ CR2 + cyclin E + Ha-RasV12 were capable of relatively normal proliferation, an indication that cyclin E is in fact competent of rescuing the proliferative defect seen in E1A Δ CR2 + Ha-RasV12 expressing cells. Based upon these results, we therefore tested the ability of cyclin E to rescue the E1A Δ CR2 mutation in transformation. Quite strikingly, while E1A Δ CR2 + cyclin E + Ha-RasV12 indicate a restoration of proliferative capacities, these cells were incapable of promoting anchorage-independent growth in soft agar. These results, as was also indicated in the 2003 report, indicate that while cyclin E expression may be sufficient to rescue the apoptotic pathways of cells expressing E1A Δ CR2, it is not the correct complement for the transformation defects of this mutant.

In addition to the aforementioned classical oncogene complementation assays, this project has also benefited from the studies of RNA interference (RNAi) mechanisms that are currently the focus of the Hannon lab, namely the use of short-hairpin RNA-expressing (shRNA) retroviral vectors. This strategy is a nice complement to the classical oncogene overexpression experiments originally proposed for this project in that we can study both the ability of overexpressed genes to rescue E1A defects and the effect of E1A-target gene silencing on transformation processes. As was indicated in the 2003 report of this project, we had begun testing a series of Rb hairpin constructs for the ability to rescue the proliferation and/or transformation defects of E1A Δ CR2 + Ha-RasV12. Last year, we reported that the coexpression of a shRNA retroviral construct (kind gift of Masashi Narita, Lowe Lab, CSHL) was capable of rescuing both the proliferative and *in vitro* transformation defects of the E1A Δ CR2 mutant. One caveat to these experiments is that the levels of Rb suppression by the shRNA constructs would indicate slight variation in each set of experiments. Therefore, the focus of the current work on this project is centered upon minimization of the variations in target gene suppression, and the construction and testing of additional shRNA constructs with different Rb targeting sequences and for additional E1A targets, such as p400, TRRAP and p300.

KEY RESEARCH ACCOMPLISHMENTS

TASK #1: Creation of Human Tumor Cell Models

- Complete and published, November 2002

TASK #2: Genetic Requirements of Human Cell Transformation

- E1A Δ CR2 can be functionally rescued by a short-hairpin RNA (shRNA) that targets pRb, and the morphology of cells coexpressing E1A Δ CR2 + shRNA-Rb + Ha-RasV12 or E1A Δ CR2 + shRNA-Rb + MDM2 + Ha-RasV12 indicates not only rescue of the CR2 deletion for proliferative capabilities, but also *in vitro* transformation
- E1A Δ CR2 + shRNA-Rb + Ha-RasV12 and E1A Δ CR2 + shRNA-Rb + MDM2 + Ha-RasV12 expressing human cells are being tested for the ability to promote tumorigenesis in immunocompromised mice. Preliminary data indicate that the latter combination is capable of promoting tumor formation, however a larger *n* must be tested.
- Doctoral dissertation supported by this award was successfully defended on December 22, 2003 and submitted to the Graduate Program in Genetics at the State University of New York at Stony Brook for degree conferral during the May 2004 commencement ceremonies.

REPORTABLE OUTCOMES

Doctoral Dissertation

Seger, Yvette Renée. Genetic Requirements for the Transformation of Normal Human Cells. Graduate Program in Genetics, State University of New York at Stony Brook. May 2004.

Manuscripts

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. *Cancer Cell* 2: 401-413.

Abstracts/Posters

2000

Seger, Y.R., Sun, P., and Hannon, G.J. "Genetic Requirements for the Transformation of Human Cells." DOD-Era of Hope Breast Cancer Meeting, Atlanta, GA, USA

Seger, Y.R., Sun, P., and Hannon, G.J. "Genetic Requirements for the Transformation of Human Cells." Cancer Genetics & Tumor Suppressor Genes Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

2001

Seger, Y.R., Maestro, R., Sun, P., and Hannon, G.J. "Genetic Requirements for the Transformation of Normal Human Cells." Telomeres & Telomerase Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Seger, Y.R., Maestro, R., Sun, P., and Hannon, G.J. "Transformation of Human Cells in the Absence of Telomerase Activation." AACR National Meeting, New Orleans, LA, USA

2002

Seger, Y.R., Maestro, R., Piccinin, S., Garcia-Cao, M., Blasco, M.A., and Hannon, G.J. "Transformation of Human Cells in the Absence of Telomerase Activation." Keystone Symposium on the Genomics and Genetics of Senescence and Cancer, Keystone, CO, USA

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. "Transformation of normal human cells in the absence of telomerase activation." Cancer Genetics & Tumor Suppressor Genes Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. "Transformation of normal human cells in the absence of telomerase activation." DOD-Era of Hope Breast Cancer Meeting, Orlando, FL, USA

Presentations

Seger, Y.R., Maestro, R., Piccinin, S., Garcia-Cao, M., Blasco, M.A., and Hannon, G.J. "Transformation of Human Cells in the Absence of Telomerase Activation." Keystone Symposium on the Genomics and Genetics of Senescence and Cancer, Keystone, CO, USA

Seger, Y.R. "Genetic Requirements for the Transformation of Normal Human Cells." Dissertation Defense. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.'

CONCLUSION

In conclusion, the work that has been completed since the 2003 report has focused upon testing the ability of shRNA constructs of E1A target genes to rescue the transformation defects of several E1A functional mutants. In the 2003 report, I presented preliminary work that indicated that shRNA constructs for the E1A target gene Rb were capable of rescuing the E1A Δ CR2 functional mutant, restoring both the proliferative capacity and *in vitro* transformation capabilities of this mutant when coexpressed with Ha-RasV12. We are currently trying to streamline this shRNA retroviral transduction system to produce gene silencing that is consistent throughout separate experiments. This will also help to provide more consistent *in vivo* transformation data, although preliminary experiments in immunocompromised mice indicate that cells expressing E1A Δ CR2 + shRNA-Rb + MDM2 + Ha-RasV12 are capable of promoting tumorigenesis, although a larger data set must be established. In sum, these data indicate that shRNA constructs will be a powerful tool in the analysis of oncogene and tumor suppressor networks and the promotion of tumorigenic phenotype.

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Transformation of normal human cells in the absence of telomerase activation

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Summary

Our knowledge of the transformation process has emerged largely from studies of primary rodent cells and animal models. However, numerous attempts to transform human cells using oncogene combinations that are effective in rodents have proven unsuccessful. These findings strongly argue for the study of homologous experimental systems. Here we report that the combined expression of adenovirus E1A, Ha-RasV12, and MDM2 is sufficient to convert a normal human cell into a cancer cell. Notably, transformation did not require telomerase activation. Therefore, we provide evidence that activation of telomere maintenance strategies is not an obligate characteristic of tumorigenic human cells.

Introduction

Neoplastic transformation occurs via a series of genetic and epigenetic alterations that yield a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth. For example, transformed cells show reduced requirements for extracellular growth promoting factors, are not restricted by cell-cell contact, and are often immortal (Paulovich et al., 1997; Hanahan and Weinberg, 2000). Through extensive studies of transformation processes in rodent models, it is known that tumor formation can be achieved by the activation of oncogenes and the inactivation of tumor suppressor pathways (Paulovich et al., 1997; Hanahan and Weinberg, 2000; Sherr, 1996). It has long been established that primary rodent cells can be transformed at detectable frequency by two oncogenic "hits," such as the combination of ectopic *c-myc* expression and constitutive activation of Harvey Ras (Ha-RasV12) (Land et al., 1983; Ruley, 1983). However, primary human cells have proven to be refractory to transformation by numerous combinations of cellular and viral oncoproteins, indicative of fundamental differences in requirements for transformation in human versus rodent cells (Sager, 1991;

O'Brien et al., 1986; Stevenson and Volsky, 1986; Serrano et al., 1997).

Two major hypotheses have emerged as the underlying explanation for such differences. Primary human and murine cells respond to oncogene activation via homeostatic mechanisms that are proposed to enforce tumor suppression. For example, activation of oncogenes such as *c-myc* or adenovirus E1A sensitizes primary cells to apoptosis (Debbas and White, 1993; Lowe et al., 1994; Lowe and Ruley, 1993; Harrington et al., 1994; Hermeking and Eick, 1994). Hyperactivation of the ras oncogene or flux through the ras signaling pathway induces a state of terminal growth arrest which is phenotypically similar to cellular senescence (Serrano et al., 1997). In murine cells, the latter response can be bypassed by genetic alterations, which impair the p53 response. Indeed, cells lacking p53 or p19^{ARF} can be transformed directly by activated ras (Kamijo et al., 1997; Serrano et al., 1996, 1997). In contrast, inactivation of the p53 pathway alone is insufficient to rescue human cells from ras-induced growth arrest (Serrano et al., 1997), suggesting that homeostatic responses in humans flow through multiple independent and redundant effector pathways.

A second characteristic that distinguishes primary human and murine cells is that the latter are easily immortalized (Blasco

SIGNIFICANCE

Activation of telomerase, and consequently telomere maintenance, is a common characteristic of human tumors. Existing models of human cancer cells, created by the introduction of defined genetic alterations, all include telomerase activation and/or telomere maintenance as an obligate component of the transformed phenotype. Here, we demonstrate that normal human cells can be converted into cancer cells, capable of forming tumors in immunocompromised mice, in the absence of telomerase activation or an alternative telomere maintenance strategy. This suggests that alterations in telomere biology must be viewed similarly to genomic instability as catalysts of transformation rather than as central components of the transformed phenotype.

et al., 1997a). Primary human cells rarely undergo spontaneous immortalization, indicating that the control of cellular lifespan is drastically different between these two cell types (Imam et al., 1997; Chin et al., 1999). This phenomenon can be partially attributed to telomere biology. Unlike the embryonic rodent fibroblasts which have served as common models for studies of transformation in vitro, primary human fibroblasts have relatively short telomeres and lack detectable telomerase activity (reviewed in McEachern et al., 2000).

The importance of telomerase in human tumorigenesis is supported by numerous observations. First, the majority of human tumors are telomerase-positive (Kim et al., 1994). Second, telomerase activation is sufficient to immortalize some primary human cells in culture (Bodnar et al., 1998; Counter et al., 1998; Wang et al., 1998). Third, telomerase is regulated by an oncogene, *c-myc*, which is activated in a high percentage of human cancers (Wang et al., 1998).

Previous reports have indicated that primary human fibroblasts and epithelial cells can be transformed by a defined combination of genetic elements, comprising the telomerase catalytic subunit, hTERT, the SV40 early region, and Ha-RasV12 (Hahn et al., 1999; Elenbaas et al., 2001; Hahn et al., 2002). Here we report an alternative model of human cell transformation. We show that coexpression of two oncogenes, adenovirus E1A and Ha-RasV12, is sufficient to enable primary human fibroblasts to grow in an anchorage-independent manner, a hallmark of in vitro transformation. However, this combination is insufficient to promote tumor formation in nude mice. Addition of a third oncogene, MDM2, can convert these fibroblasts into cells capable of forming tumors in vivo. Interestingly, both anchorage-independent growth in vitro and tumorigenesis in vivo occur in the absence of telomerase activation. Our results indicate that while telomerase activation is a common characteristic of human tumors, it is not an obligate element of the tumorigenic phenotype.

Results

Coexpression of E1A and Ha-RasV12 permits anchorage-independent growth

A defining characteristic of the transformed phenotype is a degree of independence from exogenous mitogenic signals. Many of these signals activate the *ras* pathway, and activating mutations of *ras* oncogenes or their upstream regulators often occur in human cancers (Barbacid, 1987; Webb et al., 1998). However, in both primary rodent and human cells, expression of the *ras* oncogene alone results in an irreversible growth arrest that is phenotypically similar to cellular senescence (Serrano et al., 1997; Lin et al., 1998). In rodent models, *c-myc* is capable of both bypassing *ras*-induced growth arrest and cooperating with activated Ha-RasV12 to transform primary cells into tumorigenic cells (Land et al., 1983). However, combined expression of *myc* and activated *ras* in normal human cells not only fails to result in transformation, but also leads to an accelerated appearance of the senescent-like phenotype (data not shown).

Whereas numerous genetic alterations have been shown to bypass *ras*-induced growth arrest in murine cells, only very few have been shown to be capable of overriding this response in normal human cells. One of these is the ectopic expression of

the adenovirus oncogene, E1A (Figure 1A) (Serrano et al., 1997; de Stanchina et al., 1998). In fact, coexpression of E1A and Ha-RasV12 provided one of the first demonstrations of transformation by cooperating oncogenes in primary rodent cells (Ruley, 1983). Therefore, we tested whether combined expression of E1A and Ha-RasV12 could transform normal human primary fibroblasts.

One characteristic feature of transformed cells is their ability to grow in the absence of anchorage and, therefore, form colonies in semisolid media. Early passage normal human foreskin fibroblasts (designated BJ) expressing E1A or Ha-RasV12 individually failed to form colonies in soft agar. In contrast, cells expressing both E1A and Ha-RasV12 were able to form colonies in soft agar with an efficiency comparable to that seen with transformed human and rodent cells (Figure 1B). For human 293T cells, virtually all plated cells gave rise to colonies, compared to a range of 10%–30% for BJ/E1A/Ha-RasV12 (for example, see Figure 1C). In general, colonies generated by BJ/ER (E=E1A, R=Ha-RasV12) contain significantly fewer cells than those generated by 293T cells within the same time period.

The role of E1A in the transformation of primary human fibroblasts

E1A is a multifunctional protein that interacts with numerous cellular proteins involved in controlling proliferation. For example, E1A can bind members of the Rb family through conserved motifs designated CR1 and CR2 (Whyte et al., 1988; Harlow et al., 1986; Whyte et al., 1989). Through these interactions, E1A is able to modulate the activity of the E2F family of transcription factors, thus controlling genes required for entry into S phase (Wang et al., 1995; Paulovich et al., 1997; Sherr, 1996). The amino-terminus of E1A binds promiscuous transcriptional co-activators, including p300 (Dorsman et al., 1995; Wang et al., 1995; Goodman and Smolik, 2000). The amino-terminus also binds to the protein complex containing p400, a SWI2/SNF2 family member, and the c-Myc/pCAF-interacting protein, TRRAP (McMahon et al., 1998; Barbeau et al., 1994; Fuchs et al., 2001). This p400 binding region has been shown to be vital for E1A-mediated transformation in mouse cells (Fuchs et al., 2001). The carboxy-terminal region of E1A binds CtBP, a cellular protein, which has been proposed to recruit histone deacetylases (Goodman and Smolik, 2000).

In order to map the regions and interactions of E1A that are essential for its ability to cooperate with Ha-RasV12 in conferring anchorage-independent growth to primary human fibroblasts, we used a series of well-characterized deletion mutants for in vitro transformation assays (Samuelson and Lowe, 1997). Cells were coinfecting with Ha-RasV12 and mutant E1A oncoproteins. While a truncated E1A protein consisting of only the amino-terminal 143 amino acids is unable to bind CtBP (Boyd et al., 1993; Meloni et al., 1999), this mutant is capable of cooperating with Ha-RasV12 for colony formation in soft agar with high efficiency (Figure 2). Expression of E1A-ΔCR2, a mutant incapable of binding pRb (Samuelson and Lowe, 1997), in combination with Ha-RasV12 invariably led to a senescence-like growth arrest. This result indicated that the interaction between E1A and Rb-family proteins is essential for transformation. Loss of the ability to bind p300 also compromised oncogene cooperation, as did deletion of residues 26–35, indicating that the ability to bind p400 is also critical. Western analysis of E1A mutants (Supplemental Figure S1 at <http://www.cancer.org/cgi/>

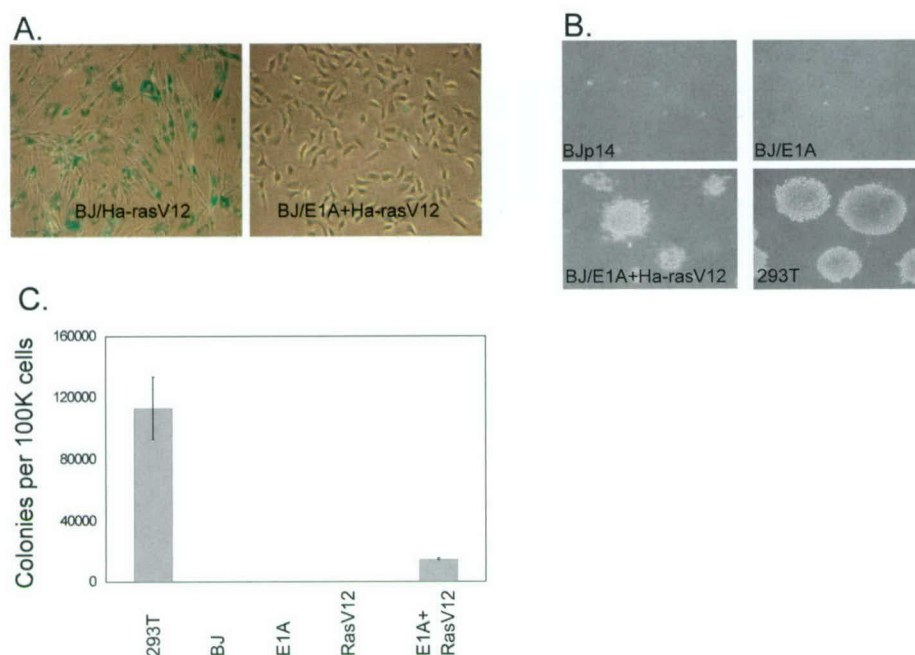


Figure 1. Transformation of normal human fibroblasts by E1A and Ha-RasV12

A: Normal human diploid fibroblasts (BJ) infected with a recombinant retrovirus that directs expression of Ha-RasV12 stain positively for expression of the senescence-associated β -galactosidase marker, whereas BJ fibroblasts coinfecting with retroviruses for E1A (12s) and Ha-RasV12 expression do not stain for this marker and continue to proliferate.

B: BJ fibroblasts were infected with retroviruses for E1A or Ha-RasV12 expression alone or in combination and assayed for colony formation in semisolid media.

C: Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype to determine the rate of colony formation. Standard error from the mean is indicated.

content/full/2/5/401/DC1) suggested that each was expressed similarly to wild-type, and thus the defects in colony formation were not due to E1A not being adequately expressed. Expression of E1A- Δ CR2 mutant was confirmed only in transient infections, since the prevalence of cell death in Δ CR2/Ras coinfecting cells prevented the establishment of a stable cell line. Considered together, these results suggest that E1A functions in human cell transformation through concerted effects on multiple cellular pathways that include Rb, p300, and p400.

E1A + Ha-RasV12-expressing cells fail to form tumors

Early and late passage BJ fibroblasts coexpressing E1A and Ha-RasV12 were tested for the ability to form tumors upon

subcutaneous injection into immunocompromised mice. A total of 49 animals were injected in both flanks in a series of five independent experiments. Subject mice were either nude, SCID (beige), or nude mice that had been irradiated as a mean to suppress residual NK (natural killer) responses (Feuer et al., 1995). From a total of 98 injections, only a single tumor formed in a nude, nonirradiated mouse (Supplemental Table S1 at <http://www.cancer.org/cgi/content/full/2/5/401/DC1>). This tumor arose after a substantially longer latency (10 weeks) than is normally observed using control cancer cell lines or transformed human 293T cells (\sim 2 weeks), suggesting the possibility that a rare additional genetic alteration may have contributed to tumor formation in this individual case. Thus, we conclude that while

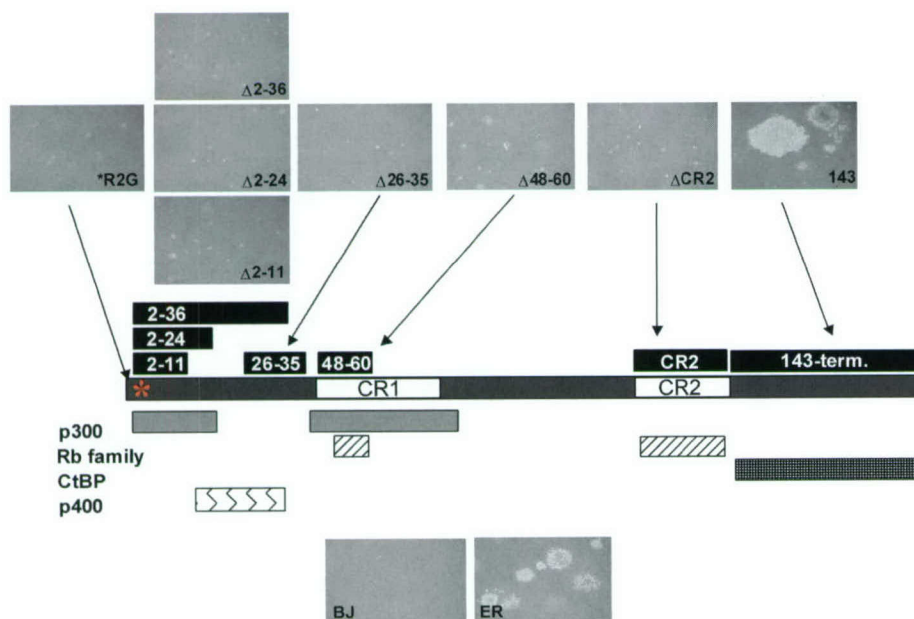


Figure 2. Functional analysis of the regions of E1A required for transformation in vitro

A series of well-characterized functional mutants of E1A were coexpressed in BJ fibroblasts with Ha-RasV12 and assayed for the ability to form colonies in semisolid media. Only the E1A-143 mutant, which is defective for the ability to bind CtBP, was capable of forming colonies in this assay, indicating that the ability of E1A to bind p300, p400, and Rb is critical for E1A-mediated transformation.

the combination of E1A and Ha-RasV12 is sufficient to permit anchorage-independent growth of normal human fibroblasts, this combination is insufficient for tumorigenesis in nude mice.

E1A, MDM2, and Ha-RasV12 transform normal human cells into tumor cells

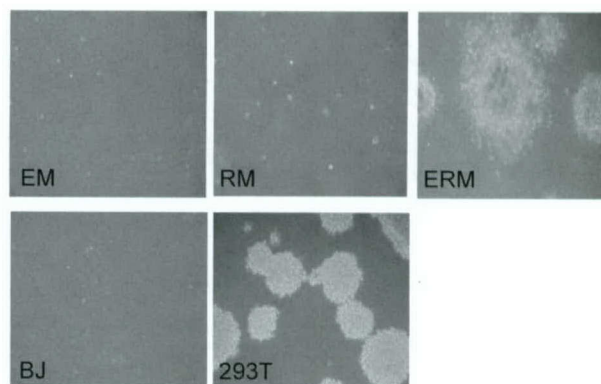
Previous studies of E1A/Ha-RasV12-mediated transformation in primary mouse embryo fibroblasts (MEF) indicated that transformation mediated by this oncogene combination was much more efficient in the absence of p53 (Lowe and Ruley, 1993). In fact, tumors arising from E1A/Ras transformed MEF become apparent only after a long latency period and frequently lack a functional p53 pathway. Interestingly, immunohistochemical analysis of the single tumor produced by the BJ fibroblasts expressing E1A/Ha-RasV12 showed a strong accumulation of nuclear p53; however, results of SSCP analysis excluded the possibility of p53 gene mutations (data not shown).

Accumulation of wild-type p53 is a common feature of human sarcoma, the type of tumor derived from fibroblast precursors. In addition, these tumors often show overexpression of MDM2 (Dei Tos et al., 1997), indicating that negation of p53 function occurs often through mechanisms other than p53 gene mutation. Notably, the tumor that resulted from the E1A/Ha-RasV12-expressing fibroblasts was negative for the expression of p19^{ARF}, an upstream regulator of MDM2 (by immunohistochemistry, data not shown), whereas the preinjection population of engineered fibroblasts expressed p19^{ARF} abundantly. Guided by these observations, we tested whether negation of the p53 pathway via enforced expression of MDM2 could contribute to the transformation of normal human fibroblasts by E1A and Ha-RasV12.

BJ cells at different passage numbers (see Experimental Procedures for details) were simultaneously coinfectd with three retroviruses that direct the expression of E1A, Ha-RasV12, and MDM2 with each retrovirus bearing a different drug selection marker. Control cells were prepared by replacing individual oncogene-expressing viruses with an empty vector bearing the same selection marker. These triple-infected populations were simultaneously coselected with puromycin, hygromycin, and neomycin for ten days and then either plated into soft agar (Figure 3) or injected into immunocompromised mice (Figure 4). Expression of the ectopically expressed oncogenes was confirmed by Western blot (not shown). Cell populations expressing E1A/Ha-RasV12/MDM2 formed colonies in soft agar with higher efficiency than BJ/E1A/Ha-RasV12 (Figure 3B). Moreover, the triple-infected cells were able to generate tumors when injected subcutaneously into immunocompromised mice (Figure 4A and Table 1). Tumors grew to a size at which the animals had to be sacrificed within a period of three to six weeks after injection, a latency comparable to that seen with control human cancer cell lines or with transformed 293T cells (Figure 4B). Tumor formation was also observed when E1A was substituted by the C-terminal deletion mutant E1A-143 (data not shown). Histological and immunohistochemical analyses of ERM-derived tumors confirmed the human origin of the tumor cell population and indicated that the neoplasias have features of sarcoma. Moreover, immunohistochemistry confirmed the widespread and strong expression of E1A, Ras, and MDM2 oncogenes (Figure 4C).

Cell populations remained polyclonal throughout drug selection in vitro and tumorigenesis in vivo, as revealed by Southern

A.



B.

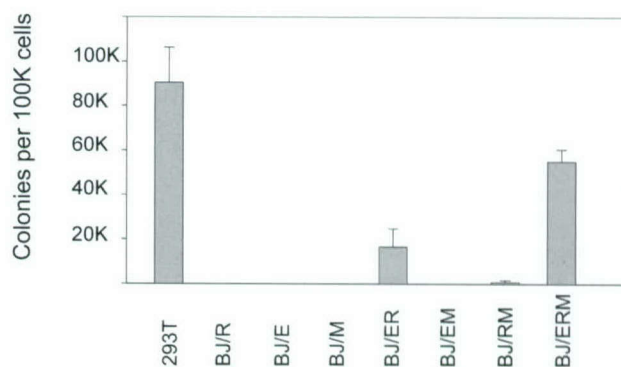


Figure 3. MDM2 cooperates with E1A and Ha-RasV12 to enhance colony formation in vitro

A: BJ fibroblasts were infected with retroviruses to direct the expression of three genes, E1A (E), Ha-RasV12 (R), and MDM2 (M), alone and in combination, and plated into semisolid media. Notably, cells engineered to express all three genes simultaneously (ERM) formed robust colonies similar to those produced in the 293T control.

B: Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype in order to determine the rate of colony formation. The standard error from the mean is indicated. ERM cells form colonies at a rate greater than ER cells and more comparable to the control 293T cell line.

blotting analysis (Supplemental Figure S2 at <http://www.cancercell.org/cgi/content/full/2/5/401/DC1>). These results argue against the possibility of selection for rare genetic events during tumor formation and support the notion that the combined expression of E1A, MDM2, and Ha-RasV12 is sufficient for the transformation of normal human fibroblasts into tumor cells.

Human fibroblasts transformed by E1A/MDM2/Ha-RasV12 lack telomerase activity

Cell immortalization has been posited as a landmark occurrence in the transformation of a normal cell into a cancer cell. Indeed, most human cancers are telomerase-positive, an indirect indication that these cells have acquired a mechanism for both telomere maintenance and extension of proliferative capacity (Kim et al., 1994). In previous reports, transformation of normal human cells absolutely required activation of telomerase via ex-

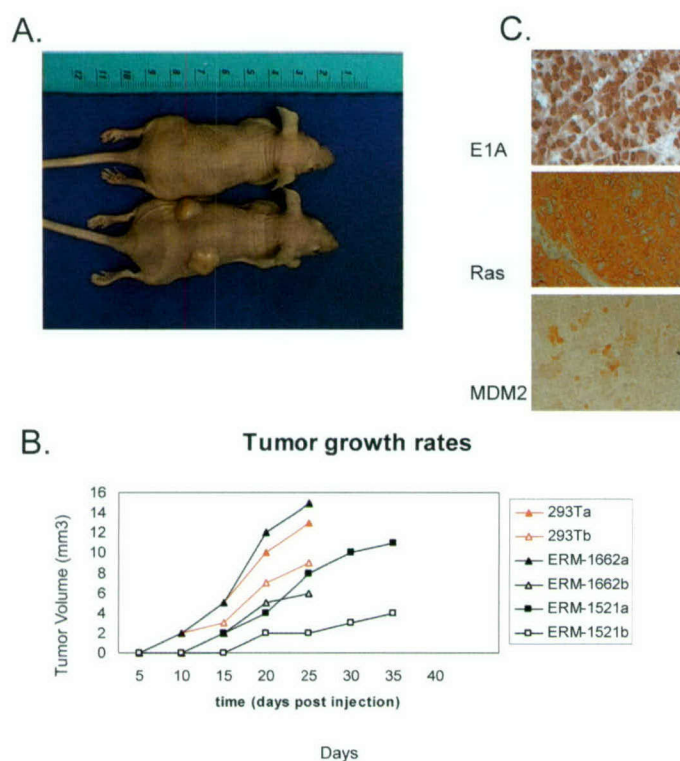


Figure 4. Conversion of BJ fibroblasts into tumor cells by combined expression of E1A, Ha-RasV12, and MDM2

A: Examples of immunocompromised mice (nude, no γ irradiation) that have been injected with either control BJ fibroblasts or BJ cells that have been engineered to express E1A (E), Ha-RasV12 (R), and MDM2 (M).

B: Tumor growth rates from two representative mice (ERM-1662 and ERM-1521) injected in both flanks (a, left; b, right) with BJ/ERM fibroblasts are compared to tumor growth rates in a mouse that had been injected in both flanks with E1A-expressing 293T cells, as indicated.

C: Immunohistochemistry staining of ERM tumor samples with antibodies to E1A, Ha-Ras, and MDM2, verifying that the tumors were in fact derived from the injected cells

pression of the limiting catalytic subunit, hTERT (Hahn et al., 1999; Elenbaas et al., 2001; Hahn et al., 2002). We previously showed that E1A, Ha-RasV12, and MDM2 were individually incapable of activating telomerase in normal human fibroblasts or epithelial cells (Wang et al., 1998). We therefore tested the possibility that we had transformed normal human cells into cancer cells in the absence of telomerase activation.

Telomerase activity was easily detected in 293T cells using the TRAP assay (Kim et al., 1994; Wright et al., 1995). As few as ten 293T cells were capable of yielding a strong positive signal in our assays. As expected, BJ fibroblasts are telomerase-negative. We similarly fail to detect telomerase activity in BJ cells that have been engineered to express E1A, Ha-RasV12, and MDM2 (BJ/ERM) (Figure 5A). We conclude that BJ/ERM cells are telomerase-negative, or contain at least 1000-fold less telomerase activity than do 293T cells at the time they are injected into immunocompromised mice.

It is interesting to note that BJ/ERM cells, although able to form colonies in soft agar and tumors in nude mice, are not immortal and, if maintained in culture for an extended period of time (40–50 generations), undergo a “crisis phase” character-

Table 1. Formation of subcutaneous tumors in nude mice by human fibroblasts expressing E1A, Ha-RasV12, and MDM2

Tumor formation in immunodeficient mice: E1A + MDM2 + Ha-RasV12

Cells	Number tumors/ number injected
293T	6/6
BJ	0/10
BJ/E1A + Ha-RasV12	0/6
BJ/E1A + MDM2	0/5
BJ/E1A + MDM2 + Ha-RasV12	34/48
BJ/E1A-143 + MDM2 + Ha-RasV12	6/6

Tumor formation: Additional human fibroblast strains

Cells	Number tumors/ number injected
BJ/E1A + MDM2 + Ha-RasV12	12/12
HSF43/E1A + MDM2 + Ha-RasV12	2/8
WI-38/E1A + MDM2 + Ha-RasV12	7/8
Detroit551/E1A + MDM2 + Ha-RasV12	7/7

For each injection, 5×10^6 cells of the indicated populations were injected subcutaneously in a volume of 100 μ l. Mice were sacrificed when the tumors reached a diameter of 1 to 1.2 cm or after 16 weeks of monitoring. The top and bottom portions of the table represent two independent series of experiments.

ized by dramatically reduced proliferation and adoption of a senescent phenotype. Few BJ/ERM cells eventually survive this phase, and these cells become telomerase-positive (Figure 5A, ERM P.C.). This behavior is suggestive of a “telomere crisis” as a consequence of the absence of a telomere maintenance program. This hypothesis is supported by an examination of telomere dynamics in BJ/ERM cells. Telomeres shrink continuously as cells are passaged in culture, reaching an average length of ~ 3 kb prior to entering a crisis phase from which the population emerges with detectable telomerase activity (Figures 6A and 6B).

Tumors derived from E1A/MDM2/Ha-RasV12 lack telomerase activity

Since ERM-engineered fibroblasts were telomerase-negative at the time of injection into mice, we were curious to ascertain the telomerase status of resultant tumors and to determine whether telomerase activation was a requirement for tumorigenesis. Telomerase activity was measured utilizing the standard TRAP assay described above on tissue sections obtained from the ERM tumors. Whereas a tissue sample from a human tumor produced a robust signal indicative of telomerase activity in a TRAP assay, lysates from ERM tumor tissues were telomerase-negative (Figure 5B). In order to verify that this negative result was not due to the presence of an inhibitory component within the tissue lysate, we performed a mixing experiment with lysate from 293T cells. When the 293T cell and ERM tumor lysates were mixed in a TRAP reaction, the result was positive, indicating that there was no inhibitory component within the tumor lysate, and thus the tumors were below detectable limits for telomerase activity (Figure 5B).

To verify the foregoing result, we used an independent experimental strategy. In human cells, and in particular in human fibroblasts such as BJ, telomerase activity correlates with the

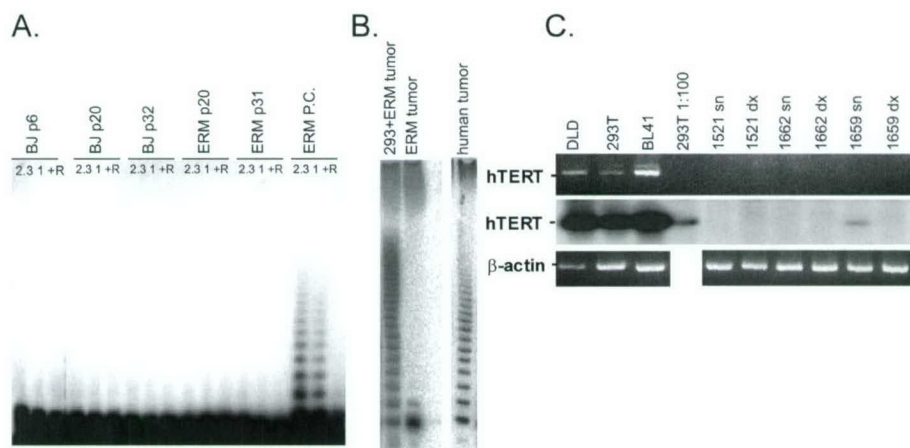


Figure 5. BJ/ERM cells are telomerase-negative upon injection into nude mice

A: The TRAP assay was used to detect telomerase activity in uninfected BJ cells and BJ/ERM cells at various passages. At the time of injection (p20), ERM cells are telomerase-negative. However, after continuous passaging in culture, these cells undergo an event similar to crisis, a point at which many cells undergo apoptosis or senescence. Cells that emerged from this crisis event become telomerase-positive (ERM P.C.). The indicated protein concentrations (μ g) of S-100 extract were used. The highest protein concentration, 2.3 μ g, was also pretreated with RNase (R) as a negative control.

B: Tumors were recovered from mice injected with BJ/ERM cells and assayed for the presence of telomerase activity using the TRAP assay. To test whether tissue extracts contained inhibitors

of any step of the procedure, we mixed lysate derived from 1,000 telomerase-positive 293T cells with the tumor extract. This produced a positive signal. The figure shows one representative tumor sample. For comparison, a similar telomerase assay performed using a mass-equivalent portion of lysate from a human breast tumor is shown.

C: RT-PCR was used to detect hTERT expression in BJ-ERM-derived tumors (tumors 1521, 1662, and 1659; left, sn⁺; right, dx⁺). Expression was tested by using two primer pairs that would direct the specific amplification of hTERT and not mouse TERT that might be present from contaminating murine cells within the tissue sample. β -actin served as an internal control. Sensitivity of the assay was increased by performing a Southern blot of the PCR reactions for hTERT. 293T, DLD colon cancer, and BL41 lymphoma cell lines were used as positive controls. 293T 1:100 represents the PCR product of 293T cell line diluted 1:100, in order to have an approximate quantification of the signal. The figure shows the result of 40 cycles of amplification for hTERT and 20 cycles for β -actin.

expression of the telomerase catalytic subunit, hTERT (Meyerson et al., 1997; Bodnar et al., 1998). We used an RT-PCR strategy to search for hTERT expression in BJ/ERM tumor specimens. Expression was tested using two independent primer pairs that were chosen for their ability to specifically amplify human TERT without amplifying mouse TERT that might be

present from contaminating murine cells in the tumor sample. β -actin mRNA served as an internal control. Ethidium bromide gel staining showed that hTERT mRNA was easily detectable in RNA derived from human cancer cell lines, but BJ/ERM tumors were negative (Figure 5C). To increase the sensitivity of our assay, we performed Southern blots of the PCR reactions. After Southern analysis, one out of six tumors analyzed, sample 1659sn, showed weak hTERT expression. This signal was detectable only with an exposure at which the signal of the positive control cells had reached saturation. The expression of hTERT in this tumor was independently confirmed by increasing the number of PCR cycles to 50 (see Figure 9B). Considered together, these data suggest that BJ/ERM cells were competent for tumor formation in the absence of telomerase activity and that activation of telomerase can occur as a late event during tumor progression.

Upon explantation into culture, BJ/ERM tumor cells, similar to late passage ERM, undergo a crisis event, which is marked by cellular senescence and apparent cell death. In contrast, explantation of tumors generated with 293T control cells did not produce a similar outcome. Instead, these cells proliferate robustly. Following this crisis event, few BJ/ERM tumor cells emerge to form a sustainable population. In contrast to early passage BJ/ERM cells and to BJ/ERM tumor samples, and similar to postcrisis late passage BJ/ERM, surviving tumor cells have become telomerase-positive (Figure 6A). The foregoing is suggestive of a "telomere crisis" possibly related to the lack of a telomere maintenance program in the tumor mass, a crisis that could be compensated by an *in vitro* selection of a cell population with activated telomerase.

In accord with this hypothesis, TRF assays and telomeric FISH confirmed that continuous telomere erosion occurred during transformation *in vitro* and tumor formation *in vivo* (Figures 6B and 7). Telomeres in early passage BJ cells averaged \sim 7 kb in length. These became depleted as BJ cells were engi-

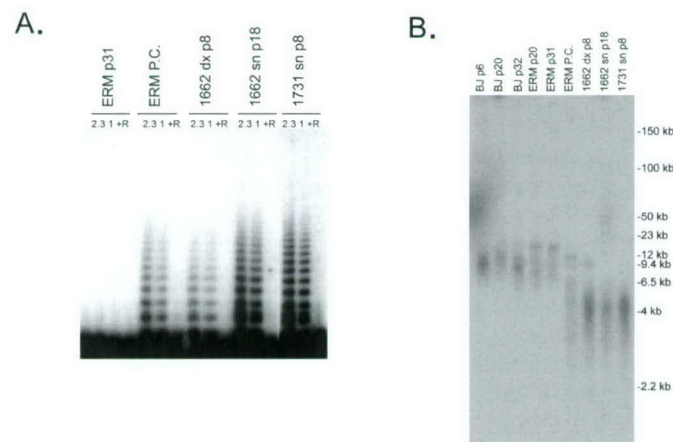


Figure 6. Telomere dynamics in ERM-transformed cells before and after tumor explant

A: The TRAP assay was used to detect telomerase activity in cells explanted from ERM tumors and compared to ERM cells that had been in continuous culture. The explanted cell lines (1662dxp8, 1662snp18, and 1731snp9) were telomerase-positive, similar to what was seen in ERM cells that had been continuously passaged in culture until they underwent crisis (ERM P.C.).

B: Telomere length in uninfected BJ, BJ/ERM, and ERM tumor explants were analyzed by TRF using pulse-field electrophoresis. As predicted, the telomeres of uninfected BJ and BJ/ERM become shorter as the cells are passaged *in vitro*. Interestingly, the telomeres of both ERM P.C. and explanted cell lines (1662dxp8, 1662snp18, and 1731snp9) had become extremely short irrespective of the fact they were telomerase-positive.

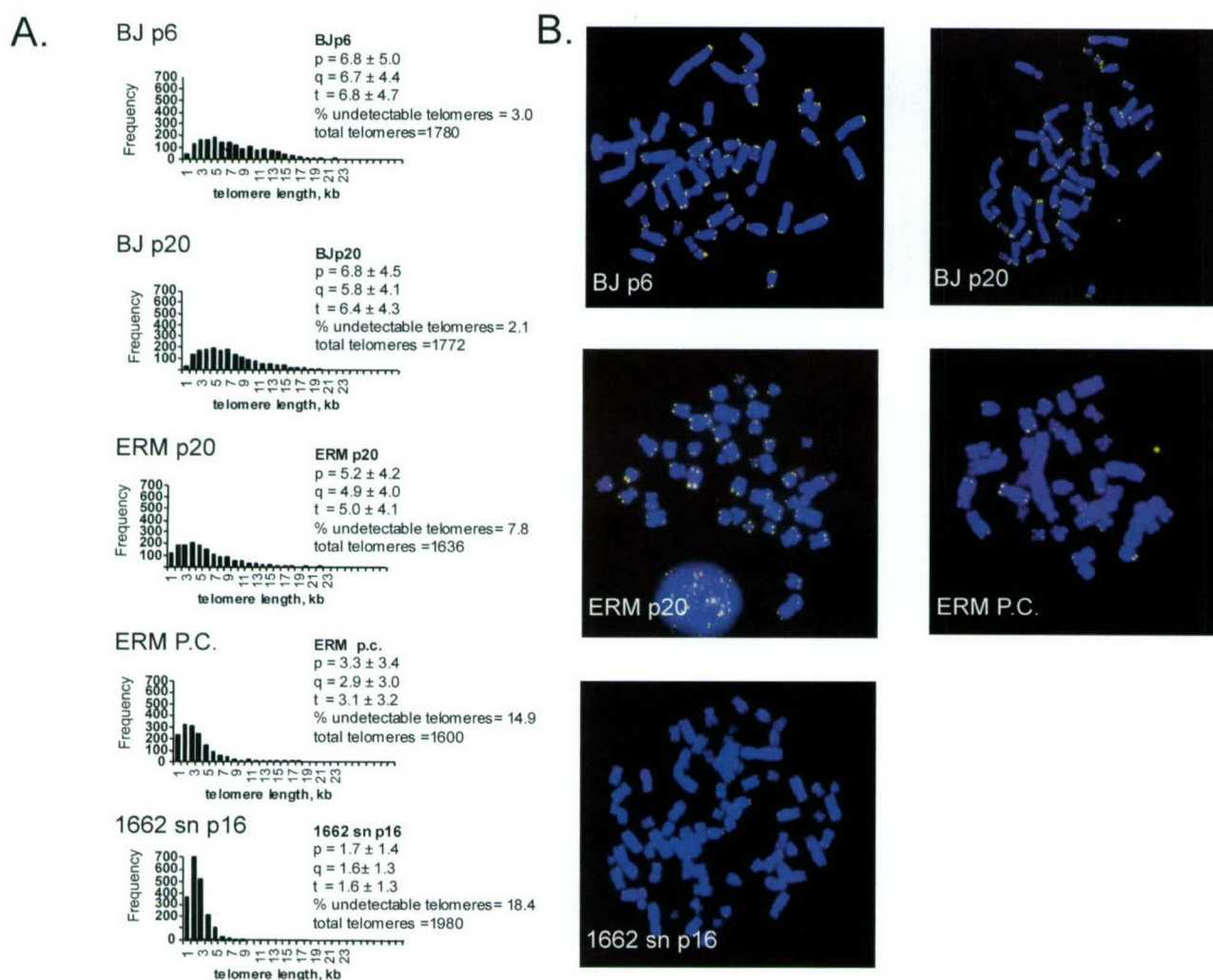


Figure 7. Quantitative FISH analysis of ERM cells preinjection and postexplant

A: Telomere length distribution of BJ, BJ/ERM, and BJ/ERM tumor cells at different passages as determined by quantitative FISH (Q-FISH) using a fluorescent Cy-3 labeled telomeric peptide nucleic acid (PNA) probe. Average telomere length in kbps and standard deviation, as well as the total number of telomeres analyzed for each cell culture, are indicated. The percentage of undetectable telomeres using Q-FISH is also indicated.

B: Representative Q-FISH images of metaphases from BJ, BJ/ERM, and BJ/ERM tumor cells hybridized with a telomeric PNA probe. We note a significant decrease in telomeric signal, as well as the increased chromosomal instability and increased aneuploidy in later passage ERM cells and in BJ/ERM tumor-derived cells, 1662sn. Blue: DAPI; yellow dots: telomeres.

needed to express oncogenes and were passaged in vitro to an average of 5 kb at passage 20 and 3.1 kb in BJ/ERM after crisis occurred in vitro. Consistent with an apparent lack of a telomere maintenance strategy, telomere depletion continued during tumor formation in vivo such that explanted cell cultures had extremely short telomeres, averaging 1.6 kb with 18% of chromosome ends lacking detectable telomeric DNA (Figure 7). These results rule out the possibility that BJ/ERM tumors have activated the recombination-based pathways of telomere maintenance (ALT) (Dunham et al., 2000; Bryan and Reddel, 1997; Hoare et al., 2001; Bryan et al., 1997). Interestingly, cells explanted from ERM tumors that had become telomerase-positive in vitro (Figure 6A) maintained their ability to form tumors in nude mice and did so at rates similar to those observed for primary ERM cells (data not shown).

The karyotypes of explanted BJ/ERM cells reveal chromosomal abnormalities characteristic of telomere depletion

As noted above, BJ cells are engineered to express E1A, Ha-RasV12, and MDM2 through simultaneous coinfection. Since these cells have not undergone prolonged expansion in the presence of any individual oncogene in culture, it is not surprising to find that the karyotypes of the engineered cells are normal prior to injection into mice (Supplemental Figure S3A at <http://www.cancer.org/cgi/content/full/2/5/401/DC1>). Examination of cells that are explanted into culture following tumor formation, however, reveals numerous chromosomal abnormalities (Figure 8, Supplemental Table S2, and Supplemental Figure S3B). In virtually every metaphase, we noted the presence of dicentric chromosomes lacking telomeres at the fusion point that apparently formed via end-to-end fusion of TTAGGG-

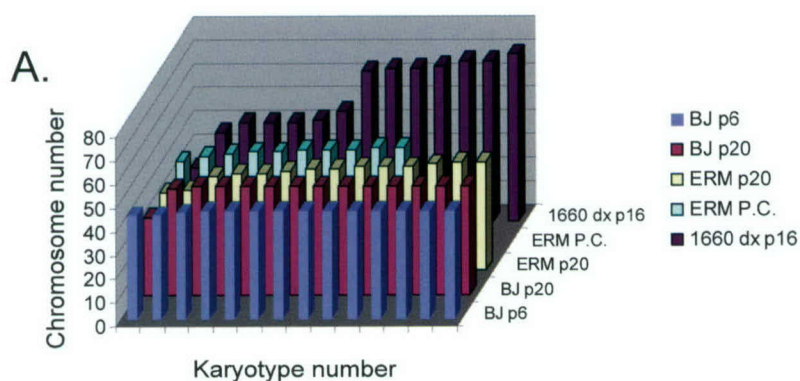
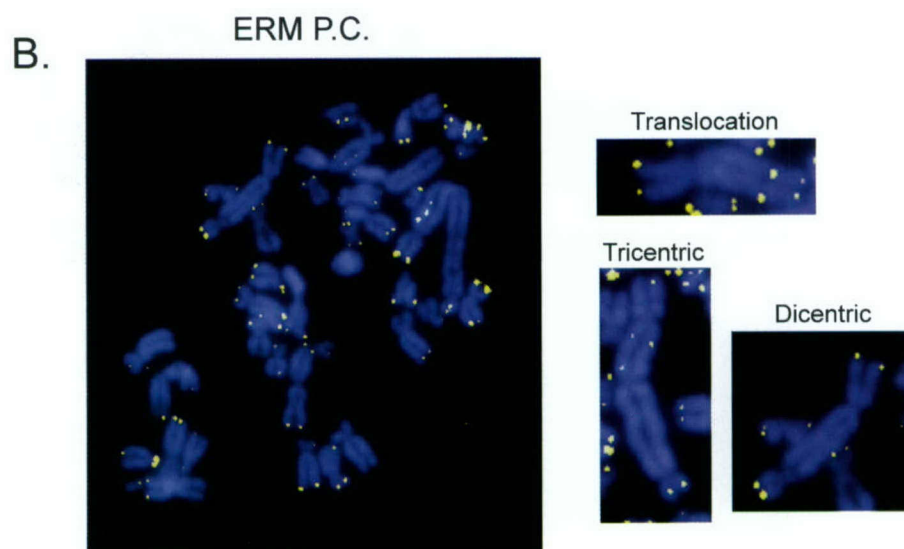


Figure 8. Comparative karyotype analysis of BJ, BJ/ERM, and BJ/ERM tumor-derived cells

A: Karyotype analysis of BJ, BJ/ERM, and BJ/ERM tumor cells at different passages is shown. For each culture, 15 metaphases were evaluated. Notice that the tumor-derived cells were severely aneuploid compared with BJ cells at two different passages. ERM p20 and ERM P.C. cells show a modest aneuploidy.

B: Representative images of cytogenetic alterations detected in metaphases from BJ/ERM explanted tumor cells after staining with DAPI and a fluorescent Cy-3-labeled telomeric PNA probe. TRANS, translocation; DICENT, dicentric; TRICENT, trivalent; blue, chromosome DNA stained with DAPI; yellow and white dots, TTAGGG repeats.



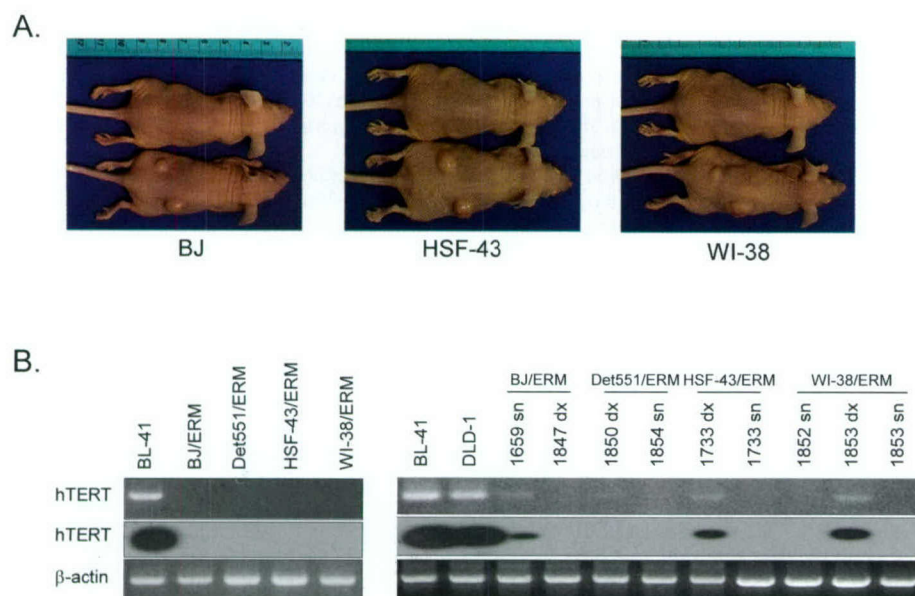
depleted telomeres. In some metaphases, we also find ring chromosomes (Supplemental Figure S3B). In addition, these cells showed a very marked aneuploidy as indicated by aberrant number of chromosomes in more than 50% of the metaphases analyzed, also in agreement with aberrant mitosis as a consequence of severe telomeric dysfunction (Figure 8). These types of genetic abnormalities are a characteristic outcome of telomere depletion and are similar to those seen in the karyotypes of *Terc*^{-/-} mice (Blasco et al., 1997b; Nanda et al., 1995). Considered together, the results of telomerase detection assays, telomere restriction fragment analyses, and cytogenetic examination of explanted tumor cells strongly suggest that combined expression of E1A, Ha-RasV12, and MDM2 is capable of transforming normal human cells into human tumor cells in the absence of direct telomerase activation or alternative mechanisms of telomere maintenance.

Multiple human primary fibroblasts can be transformed by coexpression of E1A/MDM2/Ha-RasV12

In order to ascertain whether the transforming potential of the ERM combination relied on the relatively long telomeres found in early passage BJ fibroblasts, populations of cells at different passage numbers were infected and tested for anchorage-inde-

pendent growth and tumor formation (see Experimental Procedures for details). In all the conditions tested, both early and late passage BJ/ERM invariably produced colonies in soft agar and tumors in immunocompromised mice.

Finally, to verify that E1A/MDM2/Ha-RasV12-mediated transformation is not unique to BJ fibroblasts, we assessed the validity of our transformation model in several additional human primary fibroblasts, including HSF43, WI-38, Detroit 551, and SF68 as well as in human primary mesodermal cells, HMSC. Upon coexpression of E1A/MDM2/Ha-RasV12, all were capable of anchorage-independent growth in soft agar (not shown). Efficiencies of colony formation and rates of colony growth were similar to those seen with BJ/ERM cells. Furthermore, these triple-infected fibroblasts were capable of tumor formation when injected into immunocompromised mice (Table 1 and Figure 9A). Similar to BJ/ERM, HSF43/ERM, WI-38/ERM, and DET551/ERM fibroblasts were telomerase-negative and the majority of the tumors derived from these cells failed to show telomerase expression by hTERT-RT-PCR analysis. Only 2 out of 8 tumors tested showed a faint hTERT band following 50 cycles of amplification (Figure 9B). This confirms that also in HSF43, WI-38, and DET551 fibroblasts, the ERM combination does not require telomerase activation to confer tumorigenic potential.



Discussion

Primary rodent cells and animal models have made invaluable contributions to our understanding of neoplastic transformation and of the biology of oncogenes and tumor suppressors. However, it is clear that these models do not perfectly recapitulate the process of tumor development in humans. An early indication of this fact was the inability of human cells to become transformed by the same combinations of oncogenes that could transform a variety of normal rodent cells. Recently, the ability to elicit transformation via specific genetic manipulations was extended to normal human cells (Hahn et al., 1999; Elenbaas et al., 2001; Hahn et al., 2002). This has created the opportunity for the development of a variety of defined human cancer models to be used for a detailed study of the cellular pathways that are required for the transformation of normal human cells, and ultimately, to an understanding of any differences in requirements for the transformation of human cells versus those of model organisms. Such information could provide critical insights as rationally designed anticancer therapies move from successful applications in animal models to use in humans.

Here we report that primary human fibroblasts can be transformed into cancer cells by combined expression of the adenovirus E1A, Ha-RasV12, and MDM2. As in previous models of human cell transformation, we make use of a combination of viral and cellular oncoproteins that act in a transdominant fashion to alter cellular physiology and achieve tumorigenic growth. In accord with previous reports, we show that transformation requires negation of both the Rb and p53 tumor suppressor pathways. Through genetic analyses, we have also identified requirements for interaction with p300 and p400. Both of these cellular proteins are also targeted by SV40 large T-antigen, which is a critical element of the transformation model reported by Weinberg and colleagues (Hahn et al., 1999; Elenbaas et al., 2001). However, recent reports suggest that these are not critical functions of large T, at least in the presence of small t-antigen (Hahn et al., 2002).

One striking difference between our results and those reported previously is that in our transformation model, we find no requirement for telomerase activation to achieve either anchorage-independent growth in vitro or tumor formation in vivo. In fact, consistent with their lack of telomerase or other telomere maintenance strategies, our in vitro-engineered tumor cells show continuous erosion of telomeric repeats. This ultimately leads to genetic instability that is typified by our observation of numerous chromosome end-to-end fusions and pronounced aneuploidy in cells explanted from tumor tissue.

The majority of human cancer cells are reported to be telomerase-positive (Kim et al., 1994), and this is long been considered a strong indication that the ability to maintain telomeres is an important step in the development of human cancer. However, it is still debated whether the widespread presence telomerase activity in human tumors is a reflection of a selective expansion of a telomerase-positive stem cell or a selection for a mechanism of telomere maintenance during cancer progression.

Our results are consistent with a model in which telomere maintenance is not essential for transformation, per se, but instead serves as a catalyst of tumorigenic conversion and tumor progression. Mouse models have shown that alterations in telomere biology may contribute to tumorigenesis in two ways. We and others have previously reported that telomere shortening triggers growth arrest and/or apoptosis, as well as chromosomal end-to-end fusions, leading to premature aging phenotypes in the context of the telomerase-deficient mice. These phenotypes can be rescued by telomerase activation (Lee et al., 1998; Herrera et al., 1999, 2000; Samper et al., 2001). Thus, telomere shortening during the presumably prolonged course of natural tumor development may antagonize tumorigenesis. Indeed, *Terc*^{-/-} mice are more resistant to chemical carcinogenesis and to spontaneous tumor development in both p16/p19ARF and APC^{min} mutant backgrounds (Greenberg et al., 1999; González-Suárez et al., 2000; Rudolph et al., 2001). However, exhausted telomeres can also compromise chromosome

integrity. In fact, in a p53^{+/-} background, Terc^{-/-} mice show higher levels of chromosomal instability and a higher incidence of cancer, suggesting that telomere depletion can actually act as a prooncogenic factor under certain conditions (Chin et al., 1999).

The data presented here suggest that, while changes in telomere biology are very likely to be important for the course of natural tumor development, telomere maintenance is not an absolute requirement for the creation of human cancer cells by acute alteration of oncogenes and tumor suppressors. Rather, in our human transformation model, the activation of telomere maintenance strategies becomes important only during prolonged expansion of tumor cells to restore genomic stability to an extent that permits cell survival. Our data are in agreement with observations in a number of human cancers where the frequency and intensity of telomerase activation correlates with tumor grade/stage (Chadeneau et al., 1995; Hiyama et al., 1995; Albanell et al., 1997; Tang et al., 1998; Ebina et al., 1999; Yan et al., 1999). Indeed, our ERM system can be considered as a human complement to a mouse model recently developed by DePinho and colleagues. They have shown that the absence of telomerase in Terc^{-/-}Apc^{min} mice increases the frequency of early stage intestinal adenomas but decreases the multiplicity and size of later stage lesions. They further suggest that chromosome instability arising from telomere depletion may promote early stage carcinogenesis, but that acquisition of telomere maintenance strategies is important for tumor progression (Rudolph et al., 2001).

Using oncoprotein mutants and genetic complementation, we find that inactivation of the Rb and p53 tumor suppressor pathways is critical for this transformation process. Furthermore, we find that the ability of E1A to target p300 and p400 is essential for its ability to function as a human oncogene. It will also be of interest to determine whether MDM2 contributes to the transformation of human cells solely through its ability to antagonize p53 or also via effects on additional cellular pathways.

The war on cancer is predicated on the notion that increased understanding of the biology of cancer cells might reveal an "Achilles heel" that can be exploited as an effective and specific therapeutic target. The use of rodent cell culture and of animal models have been the most informative vehicles in the drive toward this goal. However, the availability of defined human cell transformation models will allow us to build toward a complete understanding of the biological pathways that must be altered to achieve tumorigenic conversion of normal cells.

Experimental procedures

Cells

The following human primary cells were used for the experiments described in the text. We defined passage number 1 as the passage at which the cells were provided from ATCC. All the cells were maintained at a subcultivation ratio of 1:2 every 3 days.

BJ are normal human foreskin fibroblasts (ATCC catalog CRL-2522). They were provided by ATCC at population doubling 22 and are described to have the capacity to reach 80 ± 10 PD. In our hands, these cells cease proliferation at around passage 45. Most of the experiments described in the text were done using BJ infected at passage 16 and injected at passages 20, 25, and 30. However, experiments were also performed at earlier and later passages. In particular, BJ cells were infected at passage 8, 16, and 22. Cells infected at passage 8 were injected into mice at passage 12 and 20.

Cells infected at passage 16 were injected at passage 25 and 30, while cells infected at passage 22 were injected at passage 25 and 33.

WI-38 are normal embryonic lung fibroblasts (ATCC catalog CCL-75). These cells have a finite lifespan of 50 ± 10 PD and were provided by ATCC at PD24. These cells were infected at passage 6 and 10 and injected into mice at passage 14, 15, and 18.

Detroit 551 are normal human fetal fibroblasts (ATCC catalog CCL-110) provided by ATCC around PD 10 and reported to have a finite lifespan of 25 additional serial passages. These cells were infected at passage 6 and 10 and injected into mice at passage 14–15 and 18.

HSF43 are normal human foreskin fibroblasts originally isolated at the Los Alamos National Laboratory. The original PD of these cells was unknown but in our hands they cease proliferation after 40–50 passages. These cells were infected at passage 17 and 20 and injected at passage 24 and 27.

HS68 are normal human foreskin fibroblasts (ATCC catalog CRL-1635) that were received from ATCC at PD 16. These cells can be propagated for 40–50 passages. They were infected at passage 11 and 17.

HMSC 7214 are normal human mesenchymal stem cells obtained from Poietics-BioWhittaker at passage 2 (catalog PT-2501). They were derived from a 19-year-old Caucasian male and their lifespan has not been determined. These cells were infected at passage 6.

Cell culture conditions

BJ normal human foreskin fibroblasts were maintained in minimum essential medium with Earle's salts (MEM) supplemented with nonessential amino acids (NEAA) and 10% fetal bovine serum (FBS) (Gibco BRL). 293T, Detroit 551, WI-38, HSF43, and SF68 cells were maintained in Dulbecco's modified Eagle culture medium (DMEM), supplemented with 0.01% sodium pyruvate and 10% FBS. The same lot of serum was used throughout the experiments. HMSC human primary mesodermal cells (Poietics, BioWhittaker) were grown in MSCGM synthetic medium (Poietics, BioWhittaker). All cells were cultured at 37°C in the presence of 5% CO₂. All human primary fibroblasts were maintained at a subcultivation ratio of 1:2 every 3 days.

Retroviral infection

pBABE-Puro Ha-rasV12, Wzl-Neo E1A 12s, pHygroMaRX mdm2, and corresponding empty retroviral vectors were used to singularly transfect the amphotropic packaging cell line LinX-A (Hannon et al., 1999). Transfection was performed by the calcium phosphate method. At 72 hr posttransfection, viral supernatants were collected, filtered, supplemented with 4 µg/ml polybrene, and combined in order to obtain the oncogene combinations described in the text. In cells where only one or two oncogenes were used to infect the primary cells, corresponding empty vectors replaced the omitted oncogenes so that infected cells were equally resistant to all the selection drugs used (hygromycin, puromycin, and neomycin). The proper viral mix was then used to infect human primary fibroblasts (BJ, Detroit 551, WI-38, HSF43, SF68) and human primary mesodermal cells (HMSC). After infection, cells were selected with a combination of hygromycin (50 µg/ml), puromycin (1 µg/ml), and neomycin (300 µg/ml) for 7 days. Effective infection was confirmed by Western blot analysis.

Western blot analysis

Western blotting was performed essentially as described by Harlow and Lane (1999). Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with the following mouse monoclonal antibodies: E1A-specific M58 and M73 antibody—the latter recognizes an epitope retained in all E1A mutants studied (Samuelson and Lowe, 1997); c-Ha-ras (OP23) (Oncogene Research Products); mdm2-specific antibody (4B2) (a kind gift from A. Levine); bcl2 (C2) (Santa Cruz); p53 (DO-1) (Santa Cruz). Immune complexes were visualized by secondary incubation with a sheep anti-mouse HRP-conjugated secondary antibody (Amersham). Blots were developed by enhanced chemiluminescence (Amersham).

Anchorage-independent growth

Human primary fibroblasts uninfected, infected with a control empty virus, or expressing different oncogenes (E1A, Ha-rasV12, mdm2), alone or in combination, were analyzed for anchorage-independent growth in semisolid media. Approximately 10⁵ cells were plated in 0.3% low melting point aga-

rose/growth media onto 60 mm dishes with a 0.5% agarose underlay. Fresh top agar was added weekly. Colonies were photographed after 2 weeks.

Subcutaneous tumorigenicity assay

For the tumorigenicity assays, eight-week-old immunocompromised athymic nude mice (Hsd:Athymic nude-nu, Harlan) were used. Cells (5×10^6) were resuspended in 100 μ l of PBS and injected with a 25 gauge needle into anaesthetized mice.

BJ cells infected with the E1A/ras combination were also injected into 10 nude mice γ -irradiated with 400 rad prior injection and into 6 SCID beige mice (C.B-17/lcrHsd-scid-bg, Harlan). Tumor size was monitored every 5 days. Mice were sacrificed when the tumors reached a diameter of 1–1.2 cm or after 16 weeks of monitoring.

Tumors were collected in a sterile field and minced. Tumor fragments were immediately frozen in liquid nitrogen for DNA and protein extraction and for telomerase assays. Other fragments were fixed in 10% formalin for histological and immunohistochemical examinations. Finally, fragments were finely minced, washed in PBS, and plated in culture medium for isolation of tumor cells.

Tumor morphological and histochemical examination

Formalin-fixed/paraffin-embedded or snap-frozen fragments of tumor specimens were stained with hematoxylin and eosin and with histochemical stains (PAS and PAS after diastase, Reticulum, and Masson's trichrome stain) for morphological evaluation and histochemical analyses. Immunohistochemistry was performed with the peroxidase ARK kit (DAKO Glostrup Denmark) and DAB as chromogen. The following primary antibodies were utilized: Intermediate filaments (Vimentin, pan-keratin, desmin) and other human specific monoclonal antibodies (S100 protein, EMA, CD45) were used as histogenetic markers. The expression of ectopically expressed oncogenes was determined by using monoclonal antibodies specific for MDM2 (4B2) (a kind gift from A. Levine), p21-ras (OP23) (Oncogene Research Products), and E1A (M73).

Scoring of chromosomal abnormalities

Karyotype analysis

Metaphase chromosomes preparation from explanted tumor cells and quinacrine banding (QFQ staining) were according to standard protocols.

Q-FISH

The indicated numbers of metaphases from each culture were scored for chromosomal aberrations by superimposing the telomere image on the DAPI chromosome image in the TFL-telo software (gift from Dr. Peter Lansdorp, Vancouver). End-to-end fusions can be two chromosomes fused by their p-arms (Robertsonian-like fusions) or two chromosomes fused by their q-arms (dicentric).

Clonality analysis

To confirm the polyclonality of tumor cell population, genomic DNA was extracted from parental and explanted tumor cells by conventional Proteinase K/SDS digestion. Twelve micrograms of DNA were digested with either EcoRI or BamHI, alone or in combination with XhoI or SalI, and fractionated in a 0.8% agarose gel. After transfer onto Hybond N+ membrane (Amersham), blots were hybridized with 32 P-labeled probes specific for mdm2, E1A, or Ha-ras. Membranes were hybridized overnight at 65°C in 0.2 M NaPO₄, 1 mM EDTA, 7% SDS, 1% BSA in the presence of 15% formamide. Membranes were washed twice in 0.1% SDS, 0.2 \times SSC and once in 0.1 \times SSC at 60°C, followed by autoradiography.

Telomere length measurements

Q-FISH on metaphasic chromosomes

Metaphases were prepared for Q-FISH and hybridized as described (Samper et al., 2000, 2001). To correct for lamp intensity and alignment, images from fluorescent beads (Molecular probes, USA) were analyzed using the TFL-Telo program. Telomere fluorescence values were extrapolated from the telomere fluorescence of LY-R (R cells) and LY-S (S cells) lymphoma cell lines of known lengths of 80 and 10 kb (McIlraith et al. 2001). There was a linear correlation ($r^2 = 0.999$) between the fluorescence intensity of the R and S telomeres with a slope of 38.6. The calibration-corrected telomere fluorescence intensity (ccTFL) was calculated as described (Herrera et al., 1999).

Images were captured using Leica Q-FISH software at 400 mSec integration-time in a linear acquisition mode to prevent oversaturation of fluorescence intensity and recorded using a COHU CCD camera on a Leica Leitz DMRB fluorescence microscope.

TFL-Telo software (gift from Dr. Lansdorp, Vancouver) was used to quantify the fluorescence intensity of telomeres from at least 10 metaphases of each data point. The images of metaphases from different cell cultures were captured on the same day in parallel, and scored blind.

Terminal restriction fragment analysis (TRF)

Cells were prepared in agarose plugs and digested with Mbo I for TRF analysis using pulse-field electrophoresis as described in Blasco et al. (1997b).

Telomerase assays

Telomerase activity was measured with a modified telomeric repeat amplification protocol (TRAP), as described (Blasco et al., 1997b).

Analysis of hTERT mRNA expression by RT-PCR

Analysis of human TERT expression was carried out by RT-PCR. cDNA was synthesized from 1 μ g of total RNA using random primers in a 20 μ l reaction. 1 μ l of cDNA was then used to amplify two fragments of human TERT sequence, both spanning an intronic sequence. Primers hTERT1s, 5'-TTCTTGCCTGGCTGATGAGTGT-3' and hTERT1a, 5'-AGCGTCGGCCCTCTTTCTCTG-3' were used to amplify a 330 bp fragment spanning exons 3 and 4 of the human TERT sequence. Primers hTERT2s, 5'-ACAGCACTTGAA GAGGGTG-3' and hTERT2a, 5'-GTGCCTTCACCCTCGAGG-3' were used to amplify a 210 bp fragment spanning exons 4 and 5 of the human TERT sequence. Both PCR reactions were carried out for 40 or 50 cycles with an elongation at 72°C for 30 s and annealing at 65°C. PCR products were analyzed on a 3% agarose gel. The quality of cDNA was controlled by PCR amplification of a 500 bp fragment of a β -actin transcript in a 20 cycle-PCR reaction.

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Supplemental data for Seger et al., Cancer Cell 2, pp. 401–413

Supplemental Table 1. Tumor Formation in Immunodeficient Mice: E1A + Ha-RasV12

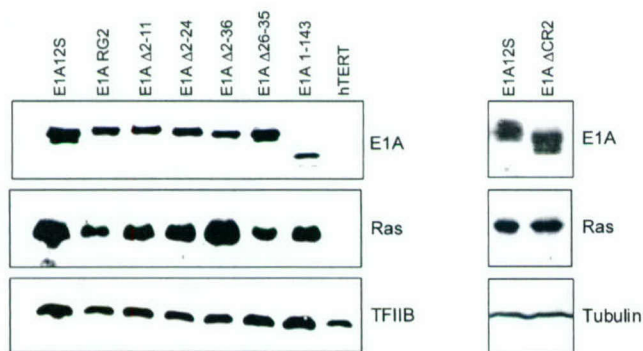
Cells	Number Tumors/Number Injections
Non-Irradiated Nudes	
293T	8/8
BJ P18	0/10
BJ/E1A + Ha-RasV12 (serial)	1*/8
BJ/E1A + Ha-RasV12 (coinfection)	0/10
Irradiated Nudes	
293T	2/2
BJ P18	0/2
BJ/E1A + Ha-RasV12 (coinfection)	0/4
SCID-beige	
293T	2/2
BJ P18	0/2
BJ/E1A + Ha-RasV12 (serial)	0/4
BJ/E1A + Ha-RasV12 (coinfection)	0/6

Subcutaneous tumor formation in immunocompromised mice by BJ fibroblasts coexpressing E1A and Ha-RasV12. BJ fibroblasts coexpressing E1A and Ha-RasV12 were unable to result in tumor formation in nude mice. There was one instance in which a single tumor formed in the flank of a bilaterally injected mouse, which is denoted as 1*. This tumor arose after a much greater latency period, indicating the possibility for the selection of a mutation in vivo.

Supplemental Table 2. Quantification of chromosomal abnormalities in BJ/ERM cells and tumors

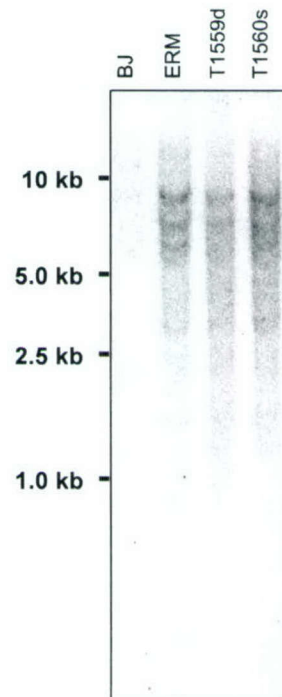
	Chromosome fragment	Dicentric	Tricentric	Translocation
BJ p6	0%	0%	0%	0%
BJ p20	0%	0%	0%	0%
ERM p20	0.16%	0.16%	0%	0%
ERM P.C.	2%	0.75%	0.5%	1.5%
1660 dx p16	0.67%	0.13%	0%	0%

Quantification of cytogenetic alterations detected in metaphases from different passages of BJ, BJ/ERM, and BJ/ERM tumor cells after hybridization with DAPI and a fluorescent Cy-3-labeled PNA probe.



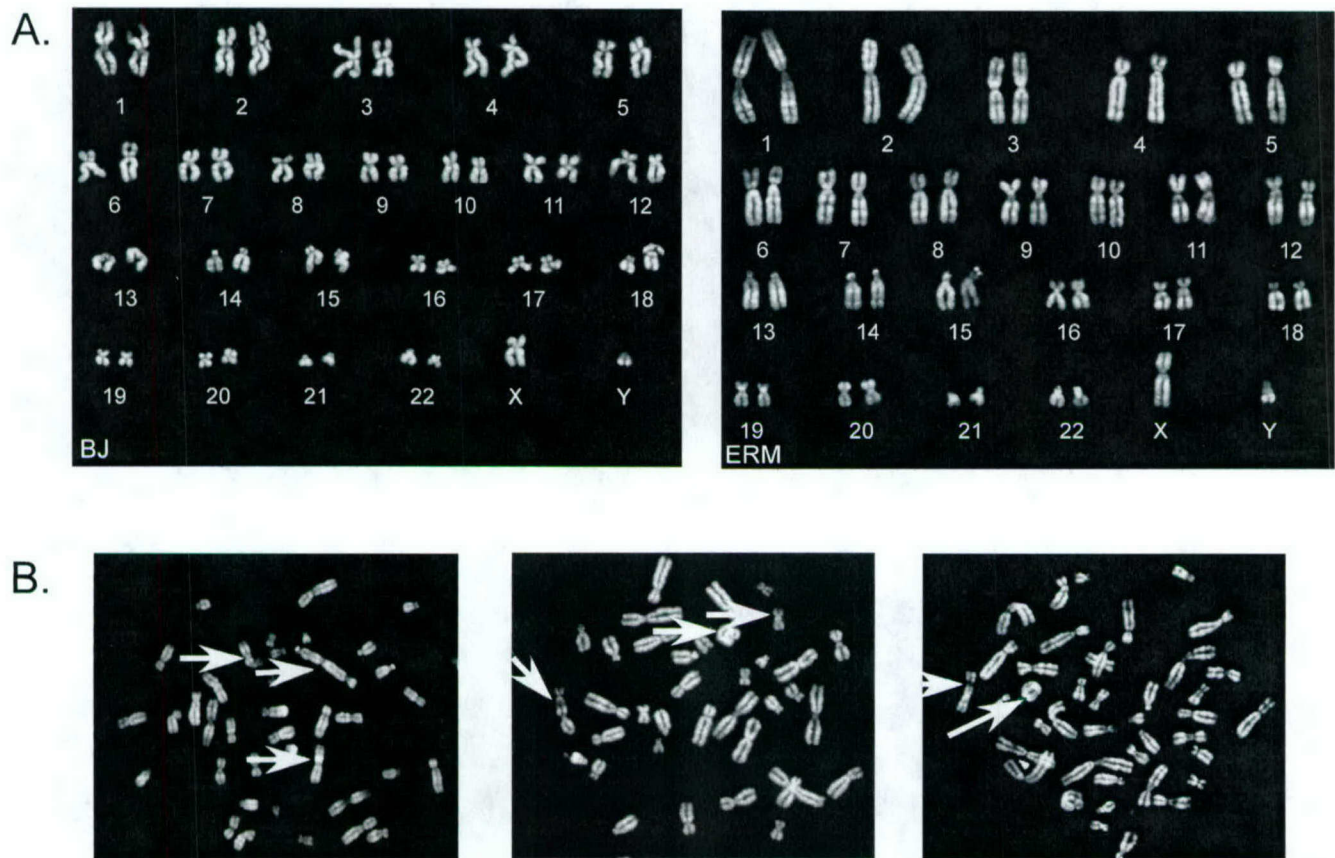
Supplemental Figure S1. Expression of E1A mutants in BJ fibroblasts

Western blot analysis indicated that the E1A mutants were expressed at levels comparable to wild-type. Expression of E1A-ΔCR2 mutant was confirmed only in transient infections (right panel), since the prevalence of cell death in ΔCR2/Ras coinfecting cells prevented the establishment of a stable cell line.



Supplemental Figure S2. Clonality analysis of BJ-ERM cells and resultant tumors

Southern analysis of BJ-ERM cells and derived tumors confirms the polyclonality of the tumors. Genomic DNA (12 μg) was digested with EcoRI and probed with a fragment corresponding to mouse mdm2 cDNA.



Supplemental Figure S3. Karyotypes of ERM cells postexplantation indicate chromosomal abnormalities characteristic of telomere depletion

A: Karyotypic analysis of normal BJ fibroblasts and BJ/ERM cells by G-banding is shown. No cytogenetic abnormalities were detected in the majority of metaphases.

B: Representative metaphases from BJ/ERM cells explanted into culture following tumor formation are shown. In virtually every metaphase, we note one or more chromosomal abnormalities, including end-to-end fusions and ring chromosomes. This is correlated with a high degree of cell death during the explantation procedure and may reflect this cell population entering a crisis phase during telomere depletion.